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Adrenergic and Adenosinergic Regulation of the Cardiovascular System in an Antarctic Icefish: Insight into Central and Peripheral Determinants of Cardiac Output

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Abstract

Icefishes characteristically lack the oxygen-binding protein haemoglobin and therefore are especially reliant on cardiovascular regulation to augment oxygen transport when oxygen demand increases, such as during activity and warming. Using both *in vivo* and *in vitro* experiments, we evaluated the roles for adrenaline and adenosine, two well-established cardio- and vasoactive molecules, in regulating the cardiovascular system of the blackfin icefish, *Chaenocephalus aceratus*. Despite increasing cardiac contractility (increasing twitch force and contraction kinetics in isometric myocardial strip preparations) and accelerating heart rate (f_H), adrenaline (5 nmol kg^{-1} bolus intra-arterial injection) did not significantly increase cardiac output ($\dot{Q}$) *in vivo* because it elicited a large decrease in vascular conductance (G_{sys}). In contrast, and despite preliminary data suggesting a direct negative inotropic effect of adenosine on isolated atria and little effect on isolated ventricle strips, adenosine (500 nmol kg^{-1}) generated a large increase in $\dot{Q}$ by increasing G_{sys}, a change reminiscent of that previously reported during both acute warming and invoked activity. Our data thus illustrate how $\dot{Q}$ in *C. aceratus* may be much more dependent on peripheral control of vasomotor tone than direct regulation of the heart.

Keywords: Channichthyidae, adrenaline, adenosine, heart rate, conductance

Introduction

Icefishes (Family: Channichthyidae) distinctively lack the expression of haemoglobin (Ruud, 1954; Sidell and O'Brien, 2006) and the high values reported for cardiac output ($\dot{Q}$) are thought of as a compensatory mechanism for the associated ~10-fold reduction in arterial carrying capacity for oxygen. However, we have recently demonstrated, with direct measurements of ventral aortic blood flow in the blackfin icefish *Chaenocephalus aceratus,* that routine $\dot{Q}$ (26.6 ml min^{-1} kg^{-1}; Joyce et al., 2018a) is much lower than the majority of previous estimates in this species (60 – 150 ml min^{-1} kg^{-1}; Hemmingsen et al., 1972; Holeton, 1970), which means that icefishes have a considerable scope in which to increase $\dot{Q}$. Indeed, during acute warming (0 to 8^{o}C) and moderate activity, the blackfin icefish substantially increased $\dot{Q}$ up to a maximum of 86.3 ml min^{-1} kg^{-1} (Joyce et al., 2018a). This increase was also associated with an up to five-fold increase in systemic vascular conductance (G_{sys}) from 15 to 75 ml kPa^{-1} min^{-1} kg^{-1} (i.e., systemic vascular resistance, R_{sys}, decreased). In the absence of haemoglobin, these large cardiovascular changes are crucial to augment oxygen transport when oxygen consumption increases (Joyce et al., 2018a) because it is not possible to greatly change the arterio-venous oxygen difference, nor is it possible to increase haematocrit, as occurs prominently in other Antarctic fishes (Axelsson, 2005; Franklin et al., 1993; Joyce et al., accepted). The aim of the present study was to address the mechanisms responsible for cardiovascular regulation in icefish. We chiefly focussed on adrenaline and adenosine, two well-established cardio- and vasoactive molecules, as potential mediators of the cardiovascular response to stressors such as warming and activity.

The importance of adrenergic modulation of cardiovascular performance in Antarctic fishes remains ambiguous. Plasma catecholamines (i.e., adrenaline and noradrenaline) are not elevated during moderate exercise in *C. aceratus,* or the red-blooded species *Notothenia coriiceps* and *Trematomus bernacchii* (Davison et al., 1995; Egginton, 1997). However, in two red-blooded Nototheniids, *Pagothenia borchgrevinki* and *Trematomus bernacchii*, plasma noradrenaline and adrenaline increased in response to severe hyperthermia (10^{o}C; Forster et al., 1998). Likewise, Whiteley and Egginton (1999) observed high catecholamine levels in both red- and white-blooded Antarctic fishes immediately following capture by trawling, suggesting adrenergic control may be

recruited *in extremis* (Whiteley and Egginton, 1999). This study nonetheless revealed a low capacity for catecholamines synthesis in Antarctic fishes (Whiteley and Egginton, 1999).

Catecholamines characteristically increase heart rate (f_H) and cardiac contractility in teleost fishes *via* direct stimulation of cardiac β-adrenoceptors, either via sympathetic autonomic innervation of the heart or humoral release into the circulation (Farrell and Smith, 2017). Nevertheless, in *in situ* perfused hearts, maximum adrenergic stimulation had little or no effect on maximum $\dot{Q}$, V_S or f_H in icefish (*C. aceratus* and *Chionodraco rastrospinosus*) (Egginton et al., submitted). Skov et al. (2009) revealed a positive inotropic effect of adrenaline in *C. aceratus,* in isometric ventricular strip preparations, while atrial preparations were insensitive to adrenaline (Skov et al., 2009). Catecholamines are also potent modulators of vasomotor tone and decrease G_{sys},, but typically increase branchial vascular conductance (G_{branch}) in teleost fishes (Pettersson and Nilsson, 1980; Sandblom and Gräns, 2017; Wood, 1974), offering the potential for dynamic cardiorespiratory coupling.

The capability of the cardiovascular system to respond to adrenergic stimulation has largely been elucidated by pharmacological interventions. Indeed, adrenaline injection in *P. borchgrevinki* elevated $\dot{Q}$, exclusively by increasing stroke volume (V_S) without changing f_H (Axelsson et al., 1994; Sandblom et al., 2012). Because ventral (P_{va}) and dorsal (P_{da}) aortic pressure increased in proportion to the increase in $\dot{Q}$, it was revealed that G_{sys} did not change and branchial conductance (G_{branch}) increased only modestly (Axelsson et al., 1994; Sandblom et al., 2012). In *T. bernacchii*, adrenaline injection increased both V_S and f_H, as well as total vascular resistance (R_{tot}, the sum of R_{sys} and R_{branch}) (Axelsson et al., 1992). In *C. aceratus*, noradrenaline injection increased P_{da} without affecting f_H (Egginton, 1997), but as this response was not measured simultaneously with blood flow the effects of adrenergic stimulation on $\dot{Q}$ and vascular conductance *in vivo* remain unknown.

Adenosine is also capable of exerting diverse cardiovascular effects when it is released from cells as oxygen demand increases or oxygen supply decrease (Mubagwa et al., 1996), such as may occur during exercise and/or warming. In this way, $\dot{Q}$ is controlled

peripherally rather than centrally by the autonomic nervous system. In fishes, including the red-blooded Antarctic notothenioid *P. borchgrevinki*, adenosine injection slowed the heart, increased G_{sys} and decreased G_{branch} (Sundin et al., 1999; Sundin and Nilsson, 1996). In both *N. coriiceps* and *C. aceratus*, exposure to high temperature (critical thermal maximum; CT_{max}) resulted in decreased cardiac ATP and increased cardiac ADP levels (O'Brien et al., 2018), which is a signature for adenosine release (Mugabwa et al., 1996), making adenosine a potential driver of the increased G_{sys} observed during warming in *C. aceratus* (Joyce et al., 2018a). In the present study, we used a pharmacological approach to provide insight into the adrenergic and adenosinergic control of the cardiovascular system in *C. aceratus*, using a combination of *in vivo* and *in vitro* experiments.

Materials and Methods

Experimental animals

Adult *Chaenocephalus aceratus* of both sexes were captured at 100-200 m depth using an otter trawl deployed from the *ARSV Laurence M. Gould* in Dallmann Bay (64°10′S, 62°35′W) and off the south-western shore of Low Island (63°24'S, 62°10'W). The fish were held on the ship in aerated sea water at ambient temperature for up to 2 days during transportation to Palmer Station, Antarctica. Full details of animal husbandry are described elsewhere (Joyce et al., 2018a). Briefly, the fish were maintained in 700 or 1700 L tanks in aerated seawater at 0 ± 1°C. The fish did not feed in captivity. The fish were allowed to recover from transportation for at least 72 hours prior to experiments. All experiments were approved and permitted by the University of Alaska, Fairbanks Institutional Animal Use and Care Committee (570217-9).

The *in vivo* data reported here were acquired from 12 fish (body mass: 0.87 ± 0.18 kg, mean ± SD) instrumented for our study on the cardiovascular effects of activity and warming (Joyce et al., 2018a). All of the data in the present manuscript were acquired approximately 12 h (overnight) after a heating protocol, during which environmental temperature had been raised to 8°C (i.e., substantially lower than CT_{max}: 12 – 14 °C (Beers and Sidell 2011; Joyce et al. 2018a; O'Brien et al. 2018)). A rapid cooling was used to

restore ambient water temperature to 0-1°C within 1-2 h. All cardiovascular variables stabilized at or close to baseline values overnight, suggesting the fish were fully recovered before the present study commenced.

In vivo pharmacological study

Surgery and Instrumentation for the in vivo *measurements*

The surgery was described previously in detail (Joyce et al., 2018a). Briefly, fishes were anaesthetised in cold (0±1°C) seawater containing MS-222 (140 mg l^{-1}) and maintained in an anaesthetized state (gills irrigated with 70 mg l^{-1} MS-222) on a surgery table. Surgery was performed in a cold room at ~2-3°C. A Transonic flow probe (4PSB or 2.5PSL) was placed around the ventral aorta to measure total cardiac output ($\dot{Q}$). The efferent branchial artery was occlusively cannulated using PE-50 with a 2F polyurethane tip to measure dorsal aortic pressure (P_{da}) and, in some of the fish, the afferent branchial artery in the same gill arch was cannulated using a PE-50 cannula for the measurement ventral aortic pressure (P_{va}). The cannulae and leads from the flow probe were sutured to the skin and the ventral incision was closed with 3-0 surgical silk. Post-surgery, the gills were irrigated with fresh seawater until voluntary ventilation resumed and each fish was then placed individually in a triangular 12.4 l respirometer.

The flow probe was connected to a Transonic flow meter (T402; Transonic Systems, USA). The cannulae were attached to pressure transducers (Medizintechnik, Kirchseeon, Germany), which were calibrated against a static water column before every experiment, and output signals pre-amplified by a Senselab 4CHAMP amplifier, (Somedic sales, Hörby, Sweden). The flow meter and 4CHAMP amplifier were connected to a PowerLab data acquisition system (ADInstruments, Castel Hill, Australia), interfaced to a computer running LabChart Pro (version 7; ADInstruments, Bella Vista, Australia).

Pharmacological Protocol

All experiments were conducted on resting fish at ambient temperature (0-1°C). Adrenaline (5 nmol kg^{-1}; equivalent to that previously used in bald rockcod (*P.*

borchgrevinki) by Sandblom et al. (2012) and less than the 10 nmol kg^{-1} used by Axelsson et al. (1994) also in *P. borchgrevinki*) was injected into the dorsal aorta (n=8). Once all parameters had returned to baseline levels, adenosine (500 nmol kg^{-1}; intermediate between levels previously used in epaulette sharks (*Hemiscyllium ocellatum*; Stensløkken et al., 2004) and rainbow trout (*Oncorhynchus mykiss*; Sundin and Nilsson, 1996), both 1 µmol kg^{-1}; and bald rockcod (*P. borchgrevinki;* 10 nmol kg^{-1}, Sundin et al., 1999) was likewise injected into the dorsal aorta (n=8). Once all cardiovascular parameters returned to baseline levels, subsequently (or in some cases independently in another cohort of resting fish) the icefish were treated with the muscarinic cholinergic antagonist atropine (n=12). When all parameters had stabilised ~25 min post-injection, the atropinised animals were subsequently treated with the β-adrenergic antagonists sotalol (N=8) or propranolol (n=4). We opted to use both β-adrenergic antagonists, in different animals, to mitigate for their possible respective side effects (e.g., Altimiras et al., 1997). All antagonists were administered as 2 mg kg^{-1} boluses injected into the dorsal aorta (Altimiras et al., 1997; Axelsson et al., 1992; Campbell et al., 2009). The adenosine but not the adrenaline injection was repeated after double-autonomic blockade (n=7).

Calculations

Stroke volume (V_S) was calculated as:

$$V_S = \dot{Q} / f_H$$

Given that central venous pressure is negligible in *C. aceratus* (Joyce et al., 2018a), we assumed it to be zero in the calculation of systemic conductance (G_{sys}):

$$G_{sys} = \dot{Q} / P_{da}$$

Branchial conductance (G_{branch}) was calculated as:

$$G_{branch} = \dot{Q} / P_{va} - P_{da}$$

Autonomic tones on f_H were calculated using the equations provided by Altimiras et al. (1997), using R-R interval (i.e., $60/f_H$):

Cholinergic tone (%) = ((R-R)cont – (R-R)atr) / (R-R)db)*100

Adrenergic tone (%) = ((R-R)db – (R-R)atr) / (R-R)db)*100

Where:

(R-R)cont = control R-R interval

(R-R)atr = R-R interval after cholinergic blockade with atropine

(R-R)db = R-R interval after double autonomic blockade (atropine and propranolol or sotalol)

In vitro myocardial preparations

The *in vitro* heart strip data used 5 additional *C. aceratus* (body mass: 1.53 ± 1.54 kg, mean ± SD) that were killed by a sharp blow to the head followed by pithing before the heart was dissected. Excised hearts were placed in ice-cold physiological saline (composed of (mM): NaCl (250), KCl (2.5), $MgCl_2$ (0.9), $CaCl_2$ (2.5), TES acid (3.1), TES sodium salt (6.1), glucose (5.6), pH 7.95-8) (Egginton et al. submitted) and carefully dissected. The saline solution was based on that previously published for marine fishes (Farrell et al., 2013) and modified to allow for the higher Na^+ concentration that characterises Antarctic teleosts (Egginton, 1994). Each fish provided one atrial strip (length: 9.7 ± 2.6 mm; mass 32.6 ± 22.4 mg, mean ± SD); also included are preliminary data from one ventricular strip preparation (length: 13.5 mm; mass 29.3 mg). Each strip was vertically suspended between a hook and metal rod attached to a force transducer (model P/N 730; Somedic SenseLab AB, Hörby, Sweden) using 4/0 surgical silk. The signal from the force transducer was preamplified using a 4CHAMP amplifier, (Somedic sales, Hörby, Sweden) and processed by a PowerLab data acquisition system and LabChart Pro v.7 (ADInstruments, Bella Vista, Australia). The preparations were immersed in 10 ml physiological saline in water-jacketed organ baths maintained at 1^{o}C for 10 min before stimulation started, then stabilised for 60 min at low tension before being stretched, over a second period of 60 min, to produce maximum force. The preparations were initially stimulated at 0.2 Hz with 15 ms pulses at 150% the threshold voltage required to elicit contraction (50 – 100 V) using a Grass S9 stimulator (Quincy, MA, USA).

During the experimental protocol, we performed a force-frequency trial, increasing stimulation frequency from 0.2 to 0.5 Hz, thus covering most of the *in vivo* f_H range for this species. The preparations were then exposed to a saturating concentration of adrenaline bitartrate (5 μM), which was given 10 min to take full effect, before another force frequency trial. This adrenaline dose exceeded the plasma concentration invoked in our *in vivo* investigation but was chosen to stimulate a maximum response *in vitro*. A preliminary experiment using one atrial and one ventricular strip preparation investigated the effects of adenosine (100 μM) (Aho and Vornanen, 2002) at the end of the adrenaline protocol (*i.e.,* still in the presence of adrenaline) to provide illustrative data until a dedicated study can be performed.

Statistical Analysis

For the *in vivo* study, all cardiovascular parameters were analysed in 20-s blocks immediately before and following the injections of adrenaline and adenosine. The effects of atropine, sotalol and propranolol (β-adrenergic blockade) were given 20 min to take full effect. A one-way ANOVA was used to investigate the cardiovascular effects of adrenaline and adenosine (both before and after double blockade) over time. Dunnett's multiple comparison post-hoc test was used to reveal significant changes from the pre-injection period (60 s prior to recording). Because adrenaline elicited complex interactions between f_H and V_S, linear regressions were used to investigate the relationship between how f_H and V_S changed (relative to pre-injection values) at 20-s intervals for the period 100 s after an adrenaline infusion. A repeated-measured one-way ANOVA and Tukey's post-hoc test was also used to compare cardiovascular parameters in control conditions, following muscarinic receptor blockade and following double autonomic blockade. An unpaired t-test was used to investigate differences in β-adrenergic tone achieved with propranolol and with sotalol.

For the *in vitro* experiments, contractile performance was analysed using twitch force, time to peak, maximum rate of contraction and the maximum rate of relaxation with the LabChart Pro Peak Analysis plug-in. Forces were normalised to cross-sectional area (assuming a density of 1.0 mg mm^{-3} and uniform thickness of the strips). A two-way

ANOVA, followed by a Sidak's post-hoc test, investigated the effects of adrenaline and frequency on contractile parameters.

Statistical analysis was performed with GraphPad Prism (v. 7.0d). Statistical significance was assigned when $P \leq 0.05$ and data are presented as means ± s.e.m, unless stated otherwise.

Results

In vivo *effects of adrenaline*

The mean cardiovascular changes in response to adrenaline are presented in Figure 1. $\dot{Q}$ initially decreased from 34.4 to 26.2 ml min^{-1} kg^{-1} but quickly recovered to above 42 ml min^{-1} kg^{-1}, although this delayed increase was not significantly higher than pre-treatment (Fig. 1a). Neither f_H (Fig. 1b) or V_S (Fig. 1c) was solely responsible for the initial decrease in $\dot{Q}$. Because both P_{da} and P_{va} increased (Fig. 1d) with the decrease in $\dot{Q}$, the decrease in f_H was most likely a hypertensive bradycardia baroreflex. The most typical response to adrenaline was transient bradycardia (Fig. 2a), although in some cases this consisted of only occasional prolonged beats (Fig. 2b), in others it lasted for several minutes (Fig. 2c). Consequently, mean f_H did not significantly change for the initial few minutes. The overall cardiovascular response was also influenced by $V_{S,}$ which tended to decrease with increasing cardiac afterload (i.e., an increase in P_{va}), with f_H and V_S changing reciprocally. Therefore, to quantify this interaction, we present the relationship between Δf_H and ΔV_S over the first 100 s following an adrenaline injection (Fig. 3). In fish displaying a strong bradycardia, V_S did not significantly decrease, i.e., V_S was presumably compensated by an increase in cardiac filling time. Subsequently, a tachycardia developed (likely a direct action of adrenaline) and f_H maximally reached 12.1 beats min^{-1}, greater than the control value of 8.8 beats min^{-1} (P<0.05). Adrenaline significantly decreased G_{sys} from 16.9 to <10.0 ml kPa^{-1} min^{-1} kg^{-1}, without affecting G_{branch}, thereby indicating systemic vasoconstriction (Fig. 1).

In vivo *effects of adenosine before and after autonomic blockade*

Adenosine injection significantly decreased P_{da} and P_{va}, and significantly increased $\dot{Q}$ and G_{sys} (Figs. 4 and 5), while G_{branch} was unchanged. $\dot{Q}$ peaked at 65.2 ml min^{-1} kg^{-1} while G_{sys} reached 64.6 ml kPa^{-1} min^{-1} kg^{-1}. f_H exhibited a delayed, but large increase from 8.3 to 14.7 beats min^{-1} (P<0.05), a response that was abolished by double autonomic blockade, which suggested that this tachycardia was likely a hypotensive baroreflex exerted through withdrawal of vagal tone and/or sympathetic drive. Indeed, injection of adenosine after double blockade elicited a small but significant bradycardia (Fig. 4d).

Muscarinic blockade (atropine injection) increased $\dot{Q}$ through an increase in f_H despite a modest decrease in V_S (Table 1). P_{da} and P_{va} increased in proportion to $\dot{Q}$, demonstrating that G_{sys} was unaffected by muscarinic blockade. In contrast, G_{branch} increased substantially in 4 out of 5 fish following muscarinic blockade (in two fish it doubled, but in one animal there was a small decrease, thus it was statistically unchanged overall). After atropinisation, β-adrenergic blockade significantly decreased f_H and increased V_S but had no other effects (Table 1), supporting the earlier suggestion that an adrenaline injection could have a direct (but slowed) chronotropic cardiac effect.

Cholinergic tone was calculated to be 103.0 ± 10.3 % and adrenergic tone 19.5 ± 1.0 %. There was no significant difference between adrenergic tonus calculated using either sotalol or propranolol (P=0.70).

In vitro *effects stimulation frequency, adrenaline and adenosine in atrial preparations*

Increasing stimulation frequency in atrial preparations revealed a negative force-frequency effect (Fig. 6A), shorter time-to-peak force (Fig. 6B) and a slowing of maximum contractile kinetics (Fig. 6C and D) (P<0.05). Adrenaline significantly (P<0.05) increased maximum isometric force by approximately 30% (Fig. 6A), a positive inotropic effect that reached statistical significance only at 0.2, 0.3 and 0.4 Hz. Adrenaline decreased (P<0.05) time-to-peak only at 0.3 Hz (Fig. 6B) but increased the maximum rate of contraction at all frequencies (Fig. 6C) (P<0.05). Adrenaline also invoked a significant (P<0.05) positive lusitropic effect at 0.2 and 0.3 Hz (Fig. 6D).

The preliminary data for adenosine on one atrial strip revealed a clear 50% decrease in maximum isometric force at all frequencies (Fig. 7A) but the ventricular strip did not respond to adenosine (Fig. 7B).

Discussion

The effects of adrenaline

The role of catecholamines in the regulation of the cardiovascular system in notothenioids, including icefishes, has proven controversial (Egginton, 1997; Skov et al., 2009; Whiteley and Egginton, 1999), in part because plasma catecholamine levels are normally unchanged during the stress associated with exercise (Egginton, 1997), unlike the more typical teleostean response (Reid et al., 1998). Nevertheless, plasma catecholamines can increase under extreme conditions, such as following capture by trawling (Whiteley and Egginton, 1999). Therefore, to provide further insight into the likely functional relevance of circulating catecholamines, our pharmacological study investigated the effects of adrenaline on the cardiovascular system of *C. aceratus*.

An α-adrenoceptor-mediated vasoconstriction of the systemic circulation is a well-established mechanism for regulating G_{sys} and central arterial blood pressure in fishes (Farrell, 1981; Wahlqvist, 1980; Wood, 1976; Wood and Shelton, 1975), including in Antarctic notothenioids (Sandblom et al., 2010, 2009). Thus, the immediate increase in both P_{da} and P_{va} as a result of a fall in G_{sys} (with no change in G_{branch}) when adrenaline was injected into the dorsal aorta of *C. aceratus* is consistent with such a mechanism. Indeed, the hypertensive response to adrenaline is also in accordance with previous brief reports for *C. aceratus* (Egginton, 1997; Hemmingsen et al., 1972). Nevertheless, the hypertension in *C. aceratus* was considerably more pronounced than that reported in *P. borchgrevinki* (Axelsson et al., 1994; Sandblom et al., 2012), but more akin to that in *T. bernacchii* (Axelsson et al., 1992), despite similar doses of adrenaline being used in all three studies. This increase in arterial blood pressure with adrenaline injections and the well-established barostatic reflex in fishes (Jones and Milsom, 1982; Randall and Stevens, 1967; Stevens et al., 1972) helps with our interpretation of the variable cardiac responses of *C. aceratus* to an injection of adrenaline.

A hypertensive bradycardia following adrenaline injection is typical of temperate fish (Helgason and Nilsson, 1973; Stevens et al., 1972; Wood and Shelton, 1980), although it was absent in the Antarctic notothenioids *P. borchgrevinki* and *T. bernacchii*, despite the fact that the former does develop bradycardia in response to the hypertension elicited by angiotensin (Axelsson et al., 1992; 1994). In *C. aceratus,* we observed a biphasic response to adrenaline, consisting of an initial decrease in $\dot{Q}$ followed by a recovery that matched or in some cases exceeded baseline conditions. The initial decline in $\dot{Q}$ was due to a combination of hypertensive bradycardia and a direct decrease in V_S (due to increased afterload). However, in animals with a pronounced bradycardia, the increased cardiac filling time compensated for the increase in afterload, meaning that V_S was maintained (Fig. 3). In Figure 3 the lines of best fit always intersect the x-axis at negative V_S, confirming that in the theoretical absence of chronotropic changes, the primary response to the increase in afterload is a decrease in V_S. This is consistent with the results of perfused heart studies that have demonstrated that icefish hearts are exceptionally sensitive to afterload (Egginton et al., submitted; Acierno et al., 1997; Tota et al., 1991).

The delayed recovery in $\dot{Q}$ we observed was probably attributable to the slowly developing positive inotropic (and chronotropic) action of adrenaline, which took 5-10 minutes to reach maximal effect in the *in vitro* cardiac strip preparations. The vascular response (increase in P_{da} and P_{va}) may also have been quicker to develop because the adrenaline bolus was injected into the dorsal aorta and hence perfused the systemic circulation before reaching the heart. The positive chronotropic action we observed contrasted with the finding that maximum adrenergic stimulation did not increase f_H in perfused *C. aceratus* hearts (Egginton et al., submitted). However, this earlier work required hearts to be perfused with a relatively high tonic adrenaline concentration during control conditions (50 nM), which may have saturated the sino-atrial β-adrenoceptors before the effects of additional adrenaline were investigated.

Skov et al. (2009) reported no effect of stimulation frequency or adrenaline treatment on contractile performance in *C. aceratus* atria, although this was not supported by the present data. This is remarkable because most previous investigations on fish atrial preparations have revealed clear positive inotropic effects of adrenergic stimulation

(Fløysand and Helle, 1994; Gesser, 1996; Keen et al., 1992; Lennard and Huddart, 1992; Meghji and Burnstock, 1984). Given the important role that atria have in filling the fish ventricle (Farrell, 1991), we re-evaluated the effect of adrenaline on *C. aceratus* atrial tissue. In contrast to Skov and colleagues, we observed a clear negative force-frequency effect and positive inotropic effect of adrenaline. The discrepancy may be ascribed to the higher concentration used in the present study (5 as opposed to 1 μM), which unequivocally demonstrated that adrenaline has the potential to modulate atrial contractility in this species. Plasma catecholamines after trawling have not been measured in *C. aceratus*, but in another icefish, *Champsocephalus gunnari,* total plasma catecholamine concentration (noradrenaline and adrenaline) slightly exceeded 1 μmol l^{-1} (adrenaline: 826 nmol l^{-1}, noradrenaline 238 nmol l^{-1}), and in another notothenioid (*Dissostichus mawsoni*), reached even higher levels (adrenaline: 2601 nmol l^{-1}, noradrenaline 841 nmol l^{-1}) (Whiteley and Egginton, 1999), suggesting that 1 μM may not have been high enough to represent the physiological extreme.

The effects of adenosine

In fish (Sundin et al., 1999; Sundin and Nilsson, 1996), as well as mammals (Mubagwa et al., 1996) and reptiles (Joyce and Wang, 2014), adenosine typically dilates the systemic vasculature. In *C. aceratus*, we confirmed that adenosine elicited a large peripheral vasodilatation, as evidenced by the several-fold increase in G_{sys} following injection. Quantitatively, the effect of adenosine on the systemic vasculature was similar to that in *P. borchgrevinki*, although we did not observe an effect on G_{branch}, in contrast to the decreased G_{branch} seen in *P. borchgrevinki* (Sundin et al., 1999).

Most striking was the a large increase in $\dot{Q}$ with adenosine infusion that peaked at 65.2 ml min^{-1} kg^{-1}, thereby exceeding peak $\dot{Q}$ evoked during moderate burst activity demonstrated previously at the same temperature (53 ml min^{-1} kg^{-1}; Joyce et al., 2018a). Before autonomic blockade, a delayed but prominent tachycardia was observed after adenosine infusion. Because this was abolished by atropine and β-adrenergic antagonists, we deduce that it was a hypotensive baroreflex (mediated by the autonomic nervous system) and not a direct effect of adenosine or other non-adrenergic non-cholinergic mechanisms. This response developed relatively slowly (> 2 min) in

comparison to that observed temperate teleosts, in which a baroreflex appears almost immediately following hypotension (Sandblom and Axelsson, 2005). This difference may be attributable to the low temperature slowing the responsiveness in the icefish, and is consistent with the slow positive chronotropic effect we observed in response to adrenaline infusion. Following double-blockade, adenosine elicited a slight bradycardia (1.3 beats min^{-1}/ 8.7% fall), which was smaller than that previously reported in *P. borchgrevinki* (~5 beats min^{-1}/ 21.7% fall) (Sundin et al., 1999).

In rainbow trout (*Oncorhynchus mykiss*), adenosine exerts a clear negative inotropic effect on atrial tissue, and a smaller negative inotropic effect on the ventricle (Aho and Vornanen, 2002; Meghji and Burnstock, 1984). This is similar to the response in other animals, including most mammals (Hollander and Webb, 1957) and reptiles (Joyce et al., 2014). However, in carp, a positive inotropic effect of adenosine has been reported in ventricular tissue (Vornanen and Tuomennoro, 1999). It was therefore instructive to perform a preliminary experiment to determine the effect of adenosine on the icefish heart, while urging caution given the low sample size. As adenosine exerted a clear negative inotropic on atrial tissue, and apparently little effect on ventricular tissue, this indicates that the increase in $\dot{Q}$ observed *in vivo* was unlikely to be a result of a positive inotropic effect on the heart. Therefore, the increase in $\dot{Q}$, in both blocked and unblocked fish, is likely attributable to a peripheral vascular effect. It has been similarly argued, by comparing the effects of exercise and vasodilators, that $\dot{Q}$ is largely determined by peripheral vascular conductance in humans (Bada et al., 2012; González-Alonso et al., 2008). The finding that large changes in $\dot{Q}$ were attained after double-blockade, in the absence of any change in f_H, is consistent with the view developed by pacing studies that the control of f_H *per se* is of little importance in determining $\dot{Q}$ (Joyce et al., 2018b; Munch et al., 2014).

The effects of dual autonomic blockade

The teleost fish heart operates under dual (i.e., cholinergic and adrenergic) autonomic control (Burnstock, 1969; Farrell and Smith, 2017; Vornanen, 2017; Wang, 2012). Cholinergic tone is especially pronounced in red-blooded Antarctic fishes, which, in conjunction with the near-freezing temperatures, results in low resting f_H (< 15 beats

min^{-1} (Axelsson et al., 1992; Campbell et al., 2009; Lowe et al., 2005; Sandblom and Axelsson, 2011)). In *C. aceratus*, Hemmingsen et al. (1972) observed that atropine elicited only a small increase in f_H from 16 to 18 beats min^{-1}, implying that cholinergic tone could be relatively weak in this haemoglobinless species. However, in ECG-instrumented fish (a minimally invasive surgery) we recently reported cholinergic tone of 55% and adrenergic tone of 12% (Joyce et al. 2018a), which are similar to those reported in red-blooded relatives (Sandblom and Axelsson 2011; Egginton and Campbell 2016). In the present study, the cholinergic tone was even higher (103.0%), whilst adrenergic tone was more similar (19.5%). Given that stress is characteristically associated with reduced or loss of cholinergic tone in fish (Sandblom and Axelsson, 2011; Wahlqvist and Nilsson, 1980), this supports our contention that by the time this protocol was conducted the fish had sufficiently recovered from the warming protocol on the previous day. The large increase in f_H following atropinisation was associated with an increase in $\dot{Q}$, although V_S decreased, likely due to the tachycardia decreasing cardiac filling time (Altimiras and Axelsson, 2004).

Conclusions

Adrenaline induces a range of cardiovascular effects including decreased G_{sys}, increased f_H, and increased atrial contractility. However, effects on the vasculature appears inconsistent with cardiovascular changes (i.e., increased G_{sys}) observed during activity and warming *in vivo* (Joyce et al., 2018a). This suggests that circulating adrenaline is of little importance in regulating cardiovascular physiology *in vivo*, and helps explain why plasma catecholamines do not increase during exercise in this species (Egginton, 1997). Sympathetic innervation of the heart may provide an alternative route for adrenergic control of cardiac performance. Although this was not detected in the red-blooded notothenid *P. borchgrevinki* (Sandblom et al., 2010), it remains to be investigated in icefishes.

Adenosine elicited a several-fold increase in G_{sys} that approached the maximum previously observed at high temperature (8°C) during activity (65 *vs.* 75 ml kPa^{-1} min^{-1} kg^{-1}) (Joyce et al., 2018a). Proof that adenosine is involved in the reported responses would require evidence that adenosinergic-receptor antagonists attenuate the activity- and

warming-induced vascular response. However, such an experiment may prove futile because other vasodilators (such as nitric oxide, bradykinin, and prostacyclin) likely act in synchrony (Hellsten et al., 2012; Joyner, 2013; Joyner and Dempsey, 2018; Lamb et al., 2018). Nevertheless, our data clearly illustrate that $\dot{Q}$ may be largely determined by peripheral factors, distinct from direct autonomic cardiac control (Guyton, 1968). It may nevertheless be fruitful for future studies to further investigate how adrenergic stimulation affects blood flow in combination with potentially sympatholytic vasodilators, as opposed to their independent effects.

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Figure Legends

Figure 1: The effects of adrenaline (5 nmol kg^{-1}) on cardiovascular parameters in *C. aceratus*. Intra-arterial adrenaline infusion is marked with an arrow (ADR). (a) $\dot{Q}$, cardiac output (n=8); (b) f_H, heart rate (n=8); (c) V_S, stroke volume (n=8); (d) P_{va}, ventral aortic pressure (n=6); P_{da}, dorsal aortic pressure (n=8); (e) G_{branch}, branchial conductance (n=6); (f) G_{sys}, systemic conductance (n=8). All values are means ± s.e.m. Asterisks denote significant (P<0.05) changes from the first (control) measurement within a given variable (repeated measures one-way ANOVA followed by Dunnett's multiple comparison test).

Figure 2. Original traces from 3 icefish depicting the variable cardiovascular effects of adrenaline. Arterial infusion is marked with an arrow (ADR). $\dot{Q}$, cardiac output; P_{va}, ventral aortic pressure; P_{da}, dorsal aortic pressure; f_H, heart rate. Fish 1 (a,d,g) represents a typical biphasic response consisting of an initial hypertensive bradycardia and subsequent tachycardia; Fish 2 (b,e,h) exhibits a near absence of bradycardia; Fish 3 (c,f,i) depicts a persistent bradycardia.

Figure 3: Mutual dependency of heart rate and stroke volume 20 – 100 seconds following adrenaline infusion. The change in heart rate (Δf_H) and change in stroke volume (ΔV_S) are calculated relative to pre-injection values for each individual fish. In animals in which heart rate increased, stroke volume fell and *vice versa*. This resulted in a universal decrease in $\dot{Q}$, despite the changes in f_H and V_S alone not being statistical significant.

Figure 4: The effects of adenosine (500 nmol kg^{-1}) on cardiovascular parameters in *C. aceratus*. Arterial infusion is marked with an arrow (ADO). (a) $\dot{Q}$, cardiac output (n=8 before double blockade, n=7 after double blockade); (b) f_H, heart rate (n=8, n=7 after double blockade); (c) V_S, stroke volume (n=8, n=7 after double blockade); (d) P_{va}, ventral aortic pressure (n=6, n=5 after double blockade); P_{da}, dorsal aortic pressure (n=8, n=7 after double blockade); (e) G_{branch}, branchial conductance (n=6, n=5 after double blockade); (f) G_{sys}, systemic conductance (n=8, n=7 after double blockade). All values are means ± s.e.m. Asterisks denote significant (P<0.05) changes from the first (control) measurement within a given variable (repeated measures one-way ANOVA followed by Dunnett's multiple comparison test).

Figure 5: The effect of adenosine on cardiovascular dynamics in a representative icefish before and after double-autonomic blockade. Intra-arterial adenosine infusion is marked with an arrow (ADO). $\dot{Q}$, cardiac output; P_{va}, ventral aortic pressure; P_{da}, dorsal aortic pressure; f_H, heart rate.

Figure 6. The effect of adrenaline (5 μM) and stimulation frequency on *in vitro* contractile performance of atrial strips from *C. aceratus*. Asterisks show significant differences after adrenaline treatment at a given frequency. Dissimilar letters represent stimulation frequency-dependent changes within a given treatment (lower case: control; capital letters: adrenaline treated; two-way ANOVAs followed by Sidak post-hoc test.). Values are mean + s.e.m. (n=5).

Figure 7. Preliminary data showing the effects of adenosine (100 μM) on *in vitro* contractile performance (force-stimulation frequency response) in one atrial and one ventricular preparation from *C. aceratus*.

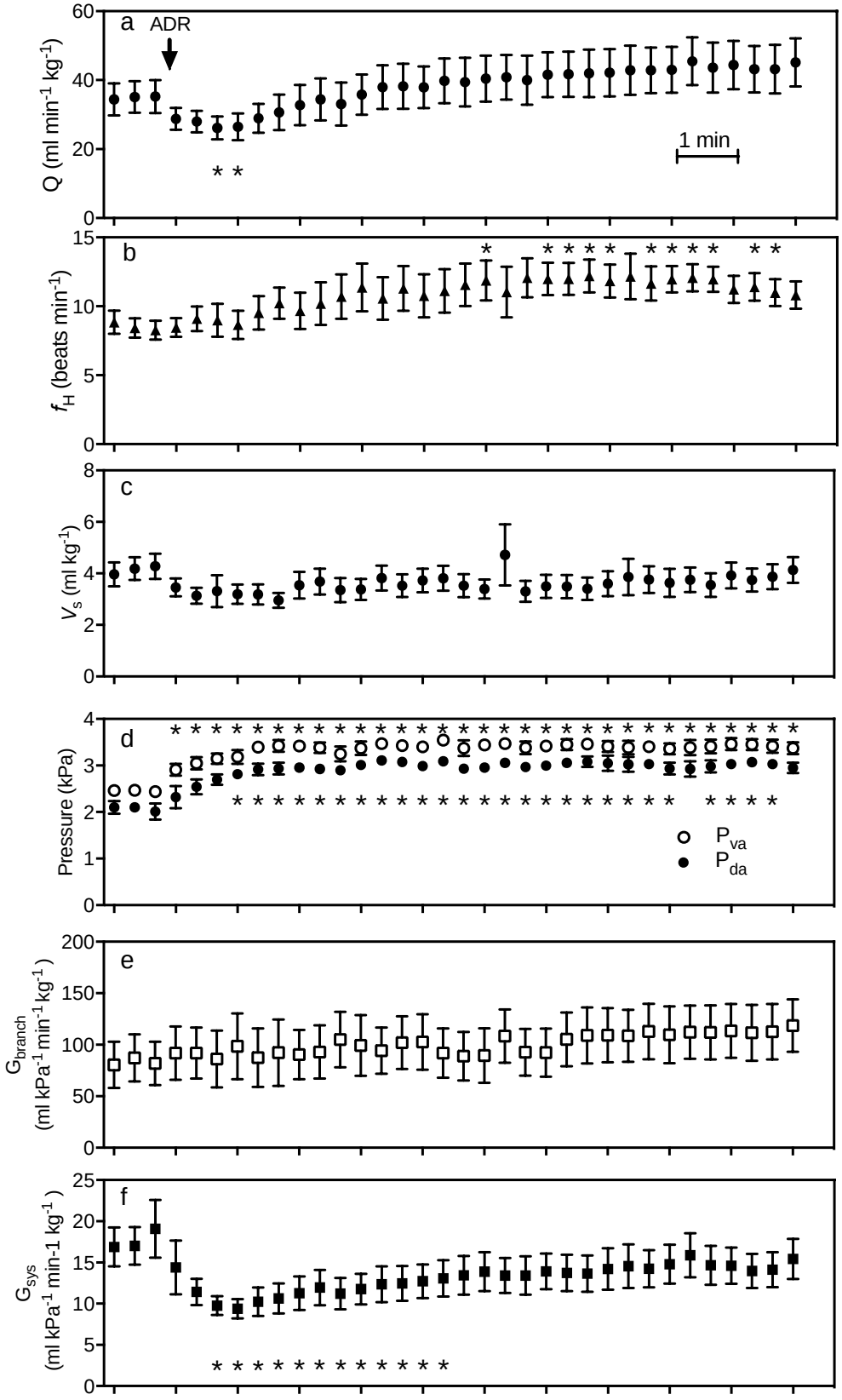
a
ADR
Q (ml min-1 kg-1)
1 min
b
fH (beats min-1)
c
Vs (ml kg-1)
d
Pressure (kPa)
Pva
Pda
e
Gbranch (ml kPa-1 min-1 kg-1)
f
Gsys (ml kPa-1 min-1 kg-1)

Figure 1.

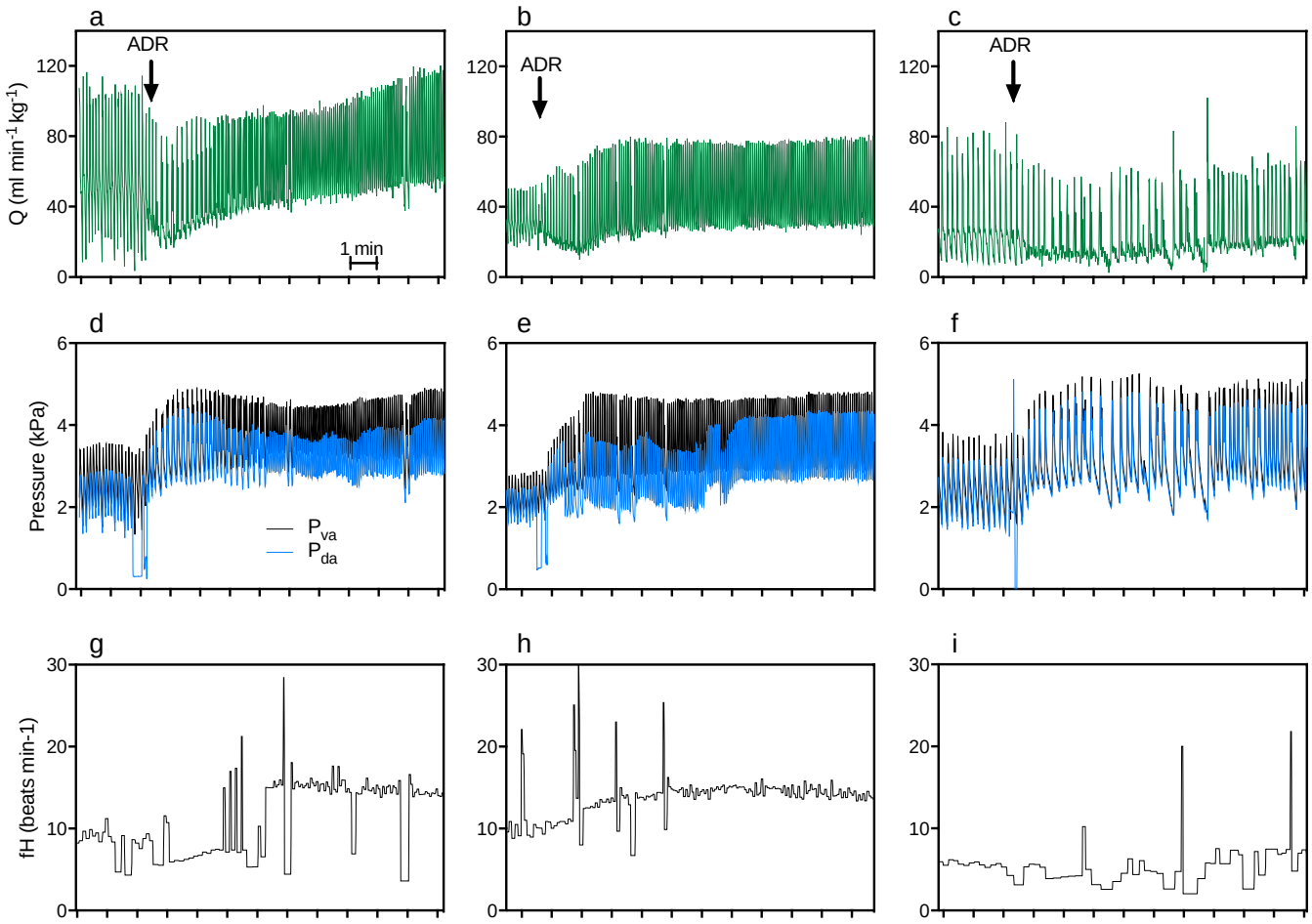
a
b
c
ADR
ADR
ADR
Q (ml min-1 kg-1)
120
80
40
0
1 min
d
e
f
Pressure (kPa)
6
4
2
0
Pva
Pda
g
h
i
fH (beats min-1)
30
20
10
0

Figure 2.

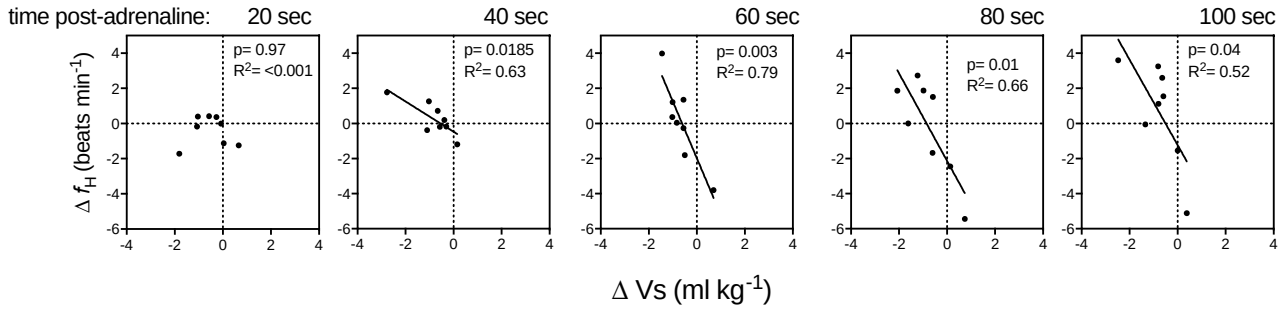
time post-adrenaline:
20 sec
40 sec
60 sec
80 sec
100 sec
p= 0.97
R2= <0.001
p= 0.0185
R2= 0.63
p= 0.003
R2= 0.79
p= 0.01
R2= 0.66
p= 0.04
R2= 0.52
Δ fH (beats min-1)
Δ Vs (ml kg-1)

Figure 3.

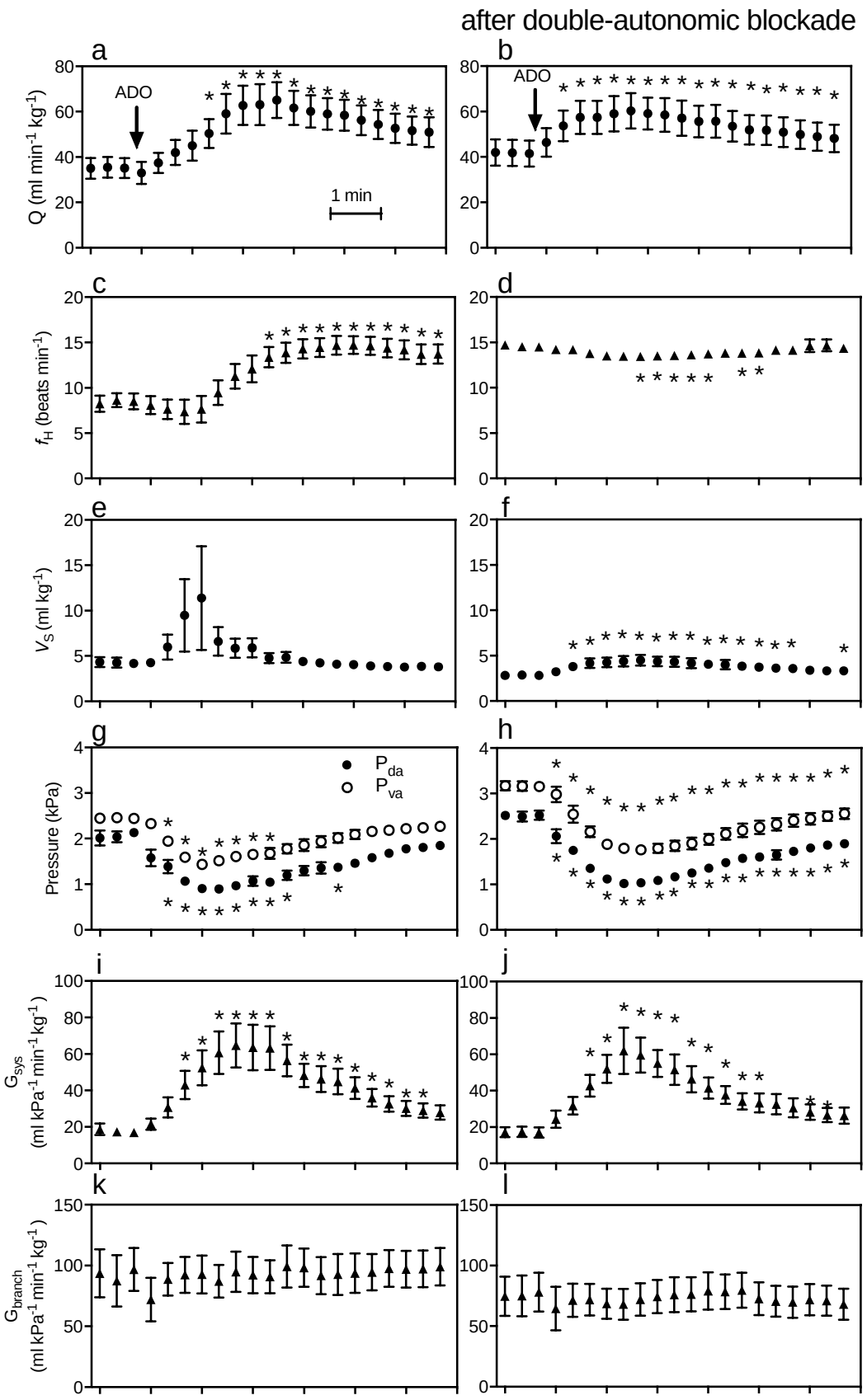
after double-autonomic blockade
a
b
c
d
e
f
g
h
i
j
k
l
ADO
ADO
1 min
Q (ml min-1 kg-1)
fH (beats min-1)
VS (ml kg-1)
Pressure (kPa)
Pda
Pva
Gsys (ml kPa-1 min-1 kg-1)
Gbranch (ml kPa-1 min-1 kg-1)

Figure 4.

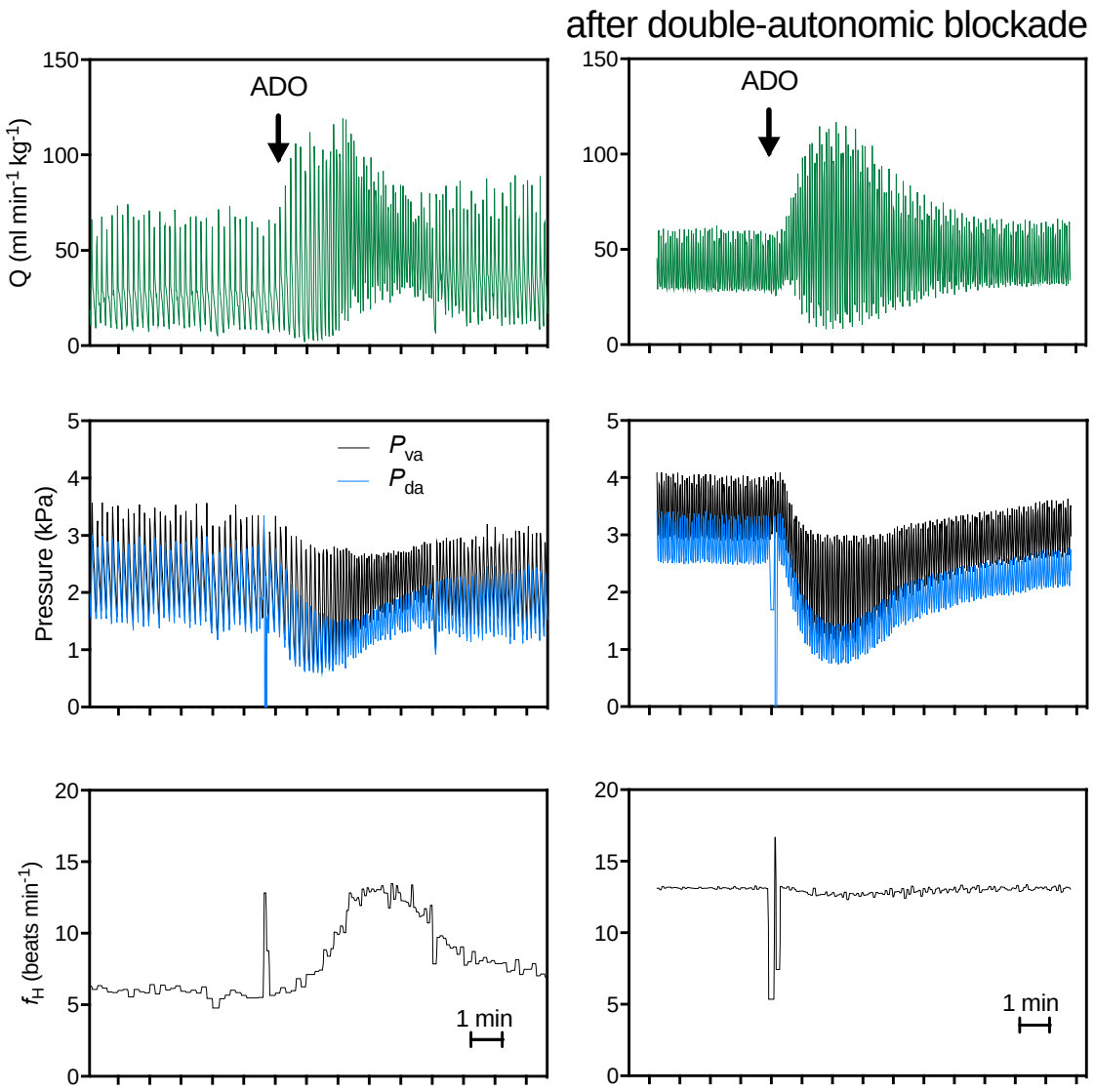
after double-autonomic blockade
ADO
ADO
Q (ml min-1 kg-1)
150
100
50
0
Pva
Pda
Pressure (kPa)
5
4
3
2
1
0
fH (beats min-1)
20
15
10
5
0
1 min
1 min

Figure 5.

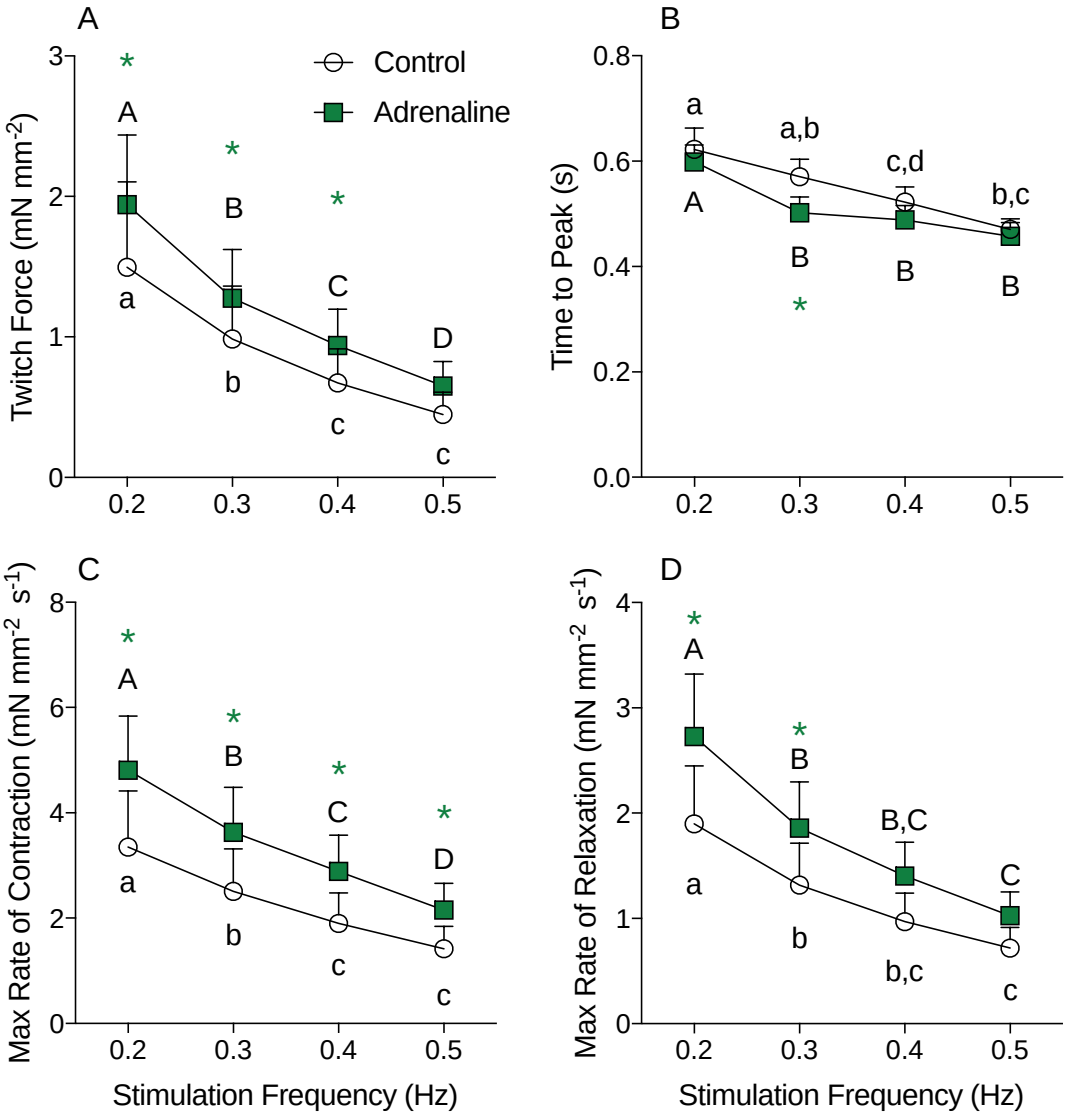
A
B
C
D
Control
Adrenaline
Twitch Force (mN mm-2)
Time to Peak (s)
Max Rate of Contraction (mN mm-2 s-1)
Max Rate of Relaxation (mN mm-2 s-1)
Stimulation Frequency (Hz)
Stimulation Frequency (Hz)

Figure 6.

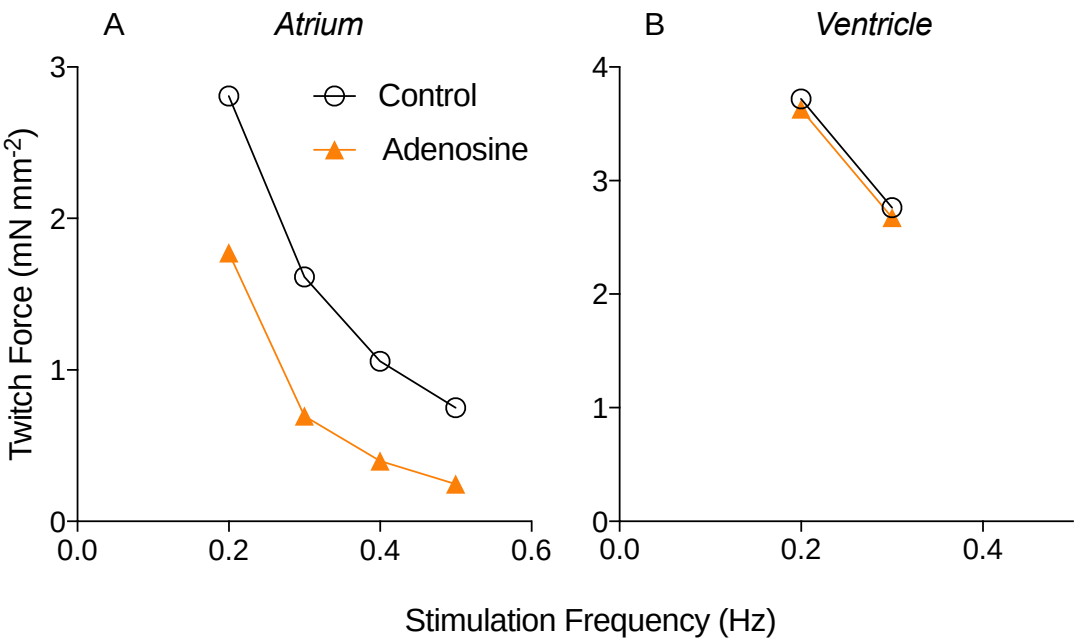
A
Atrium
B
Ventricle
Control
Adenosine
Twitch Force (mN mm-2)
Stimulation Frequency (Hz)
0
1
2
3
4
0.0
0.2
0.4
0.6

Figure 7.

	$\dot{Q}$ (ml min^{-1} kg^{-1})	f_H (beats min^{-1})	V_S (ml kg^{-1})	P_{da} (kPa)	P_{va} (kPa)	P_{cv} (kPa)	G_{sys} (ml kPa^{-1} min^{-1} kg^{-1})	G_{branch} (ml kPa^{-1} min^{-1} kg^{-1})
control	35.1 (±3.0)^a	8.5 (±0.5)^a	4.16 (± 0.35)^a	2.15 (± 0.35)^a	2.49 (± 0.06)^a	0.27 (± 0.2)^a	17.1 (± 1.7)^a	86.0 (±19.7)^a
muscarinic blockade	43.9 (±4.3)^b	18.9 (±0.6)^b	2.32 (±0.24)^b	2.84 (± 0.24)^b	3.16 (± 0.06)^b	0.00 (±0.01)^a	15.5 (± 1.5)^a	114.6 (±22.4)^a
double-blockade	42.3 (±4.3)^b	15.2 (±0.5)^c	2.78 (±0.29)^c	2.71 (± 0.29)^b	3.16 (± 0.09)^b	0.06 (±0.05)^a	16.2 (± 1.9)^a	87.0 (±16.7)^a

Table 1. The effects of muscarinic blockade (atropine; 2 mg kg^{-1}) and double autonomic blockade (atropine and propranolol or sotalol; 2 mg kg^{-1}) on cardiac output ($\dot{Q}$, n=10), heart rate (f_H, n=12), stroke volume (V_S, n=10), dorsal aortic pressure (P_{da}, n=12), ventral aortic pressure (P_{va}, n=7), central venous pressure (P_{cv}, n=3), systemic conductance (G_{sys}, n=10) and branchial conductance (G_{branch}, n=5) in icefish at ambient temperature. Dissimilar letters denote significant differences (P<0.05; repeated measures one-way ANOVA and Tukey's post-hoc test)